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Citation for published version:

Jackson, I 1988, 'A cDNA encoding tyrosinase-related protein maps to the brown locus in mouse', *Proceedings of the National Academy of Sciences (PNAS)*, vol. 85, no. 12, pp. 4392-6.
<<http://www.pnas.org/content/85/12/4392.long>>

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Proceedings of the National Academy of Sciences (PNAS)

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A cDNA encoding tyrosinase-related protein maps to the brown locus in mouse

(pigmentation/coat color/melanocytes/mutations/inbred strains)

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Communicated by Mary F. Lyon, January 25, 1988 (received for review November 10, 1987)

ABSTRACT A mouse melanoma cDNA clone was isolated by virtue of its reactivity with two antisera raised against tyrosinase (EC 1.14.18.1) from two species, hamster and mouse. The cDNA (5A) cross-hybridizes with another, pMT4 [Shibahara, S., Tomita, V., Sakakura, T., Nager, C., Bhabatosh, C. & Muller, R. (1986) *Nucleic Acids Res.* 14, 2413-2427], previously thought to encode mouse tyrosinase. Two other cDNAs, one human and one mouse, have been reported recently [Kwon, B. S., Haq, A. K., Pomerantz, S. H. & Halaban, R. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7473-7477; and Yamamoto, H., Takeuchi, S., Kudo, T., Makino, K., Nakata, A., Shinoda, T. & Takeuchi, T. (1987) *Jpn. J. Genet.* 62, 271-277] as candidates for tyrosinase, and they map at or very close to the mouse albino (*c*) locus. The proteins they encode are very similar to each other but are distinct from (although related to) the pMT4-encoded protein. Here I use recombinant inbred strains to localize pMT4 at or close to the mouse brown (*b*) locus. I suggest that the gene mapping to *c* is the authentic tyrosinase gene, whereas that mapping to *b* encodes a tyrosinase-related protein. All *b* mutations in laboratory strains are associated with the same diagnostic *Taq* I fragment, suggesting that all derive from the same original mutation. I discuss possible function(s) of the tyrosinase-related protein.

The development of the pigmentation of mouse hair is an attractive system for the study of developmental genetics (1, 2). Normal pigment formation requires correct melanocyte migration during embryogenesis, correct interaction between melanocytes and other cells, acquisition of the correct cellular and subcellular morphology, and the correct activation and function of enzymes. Mutations have been identified in all these processes.

Over past decades, many pigmentation mutants have been identified at a number of loci. Six of them, nonagouti (*a*), brown (*b*), albino (*c*), dilute (*d*), pink-eyed dilution (*p*), and piebald (*s*), are used in specific locus mutagenesis experiments (3, 4). These experiments measure rates of mutation at different loci due to various treatments and consequently produce new mutant alleles at the loci. At two loci in particular (albino and dilute) genetic analysis of putative radiation-induced deletions has allowed the study of neighboring genes that have effects on development (5, 6).

Retrovirus insertions have been used to isolate DNA from the dilute locus (7, 8) and from very close to the nonagouti locus (9, 10). Recently human and mouse cDNAs, identified as encoding tyrosinase (monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1), have been mapped at or very close to the *c* locus (11, 12). Such probes should allow the molecular basis of mutations at these three loci to be determined.

There is good biochemical evidence that tyrosinase is encoded at the *c* locus (13, 14). I set out to clone the tyrosinase gene by an immunological approach but isolated a gene that, although identified by other workers as mouse tyrosinase (15), is different from the cDNAs that map at the *c* locus (11, 12). I demonstrate here that this gene, encoding a tyrosinase-related protein (TRP), maps at or very close to the *b* locus. In addition, I present evidence that all *b* mutations in laboratory inbred strains are derived from a single progenitor.

MATERIALS AND METHODS

Materials. B16/C3 mouse melanoma cells were from Dot Bennett (St. Georges Hospital Medical School) and were cultured as described (16). Phage λ gt11 and *Escherichia coli* strains Y1088, Y1089, and Y1090 were from Yvonne Edwards (Medical Research Council Human Biochemical Genetics Unit). DNAs from mouse strains 129, DBA/2J, BALB/cBy, AKR/J, C57L/J, C57BL6/J, and recombinant inbred (RI) strains of the BXD and AKXL series were obtained from Bob Hill (this unit). DNAs from ST/bJ, NZW/LacJ, and LP/J mice were purchased from The Jackson Laboratory. DNA from *c^{3H}* mice was from Gunther Shutz (Heidelberg), and *c^{14Cos}* and the CXB RI series DNAs were from Keith Willison (Institute for Cancer Research). DNA was prepared from CBA/Ca mice and from mice of the Q stock at the Medical Research Council Mammalian Development Unit and from 101/H, C3H/HeH, and PT stock mice obtained from the Medical Research Council Radiobiology Unit. Mice carrying the *b^{cH}* and *B^H* mutations were also from the Medical Research Council Radiobiology Unit. The antibodies raised in rabbits against mouse and hamster tyrosinase were kind gifts of Vincent Hearing (National Institutes of Health) and Seymour Pomerantz (University of Maryland Medical School), respectively. The clone pMT4 was kindly donated by Shigeru Shibahara (Friedrich-Miescher-Institut).

cDNA Library. RNA was isolated from B16 melanoma cells by using the LiCl/urea method (17), and double-stranded cDNA was prepared (18) and cloned in λ gt11 (19). The library contains about 600,000 independent recombinants. It was screened according to Huynh *et al.* (19) with IgG prepared from the two sera by staphylococcal protein A-Sepharose chromatography, detected with peroxidase-conjugated pig anti-rabbit antibody, and stained with chloronaphthol in hydrogen peroxide.

DNA Preparation and Blot Hybridizations. DNA was prepared as described (ref. 20, p. 280). Restriction enzymes were used according to their suppliers' instructions. Gel electrophoresis, blotting to nitrocellulose or Hybond-N (Amersham), and hybridization were as described (ref. 20, pp. 159-162 and 383-389). DNA fragments were isolated from

low-gelling-temperature agarose gels, diluted with 3 vol of water, and radioactively labeled with ^{32}P by using the method of Feinberg and Vogelstein (21).

RESULTS

Identification of a Tyrosinase-Related Gene. To isolate putative tyrosinase clones I employed a protocol that involved duplicate screening of the B16/gt11 cDNA library. Approximately 500,000 recombinants were screened initially with RA5, a rabbit antiserum raised against hamster tyrosinase. Five clones, which gave signals of various intensities, were plaque purified and rescreened with a rabbit serum raised against mouse tyrosinase. Two of these five (1A and 5A) gave a strong signal with the second serum. The two cDNAs do not cross-hybridize. Southern blot hybridization with both cDNAs indicated that neither was deleted from DNA of the albino deletion mutants $c^{14\text{Cos}}$ and c^{3H} (data not shown) and therefore neither was encoded at the albino locus.

Shibahara *et al.* (15) reported the isolation and sequencing of a candidate cDNA for mouse tyrosinase, pMT4. Clone 5A cross-hybridizes with pMT4 and is clearly closely related to it. However, Yamamoto *et al.* (11) and Kwon *et al.* (12) recently reported the isolation of other mouse and human candidate cDNAs for tyrosinase. Kwon *et al.* demonstrated that the human candidate (mel-34) maps in the mouse at or near the albino locus. The protein it encodes shares about 34% of its amino acid residues with the pMT4-encoded protein, although these are distributed in four substantial domains that have about 50% identity between the two proteins. Inspection of the mouse sequence of Yamamoto *et al.* (Tyrs-33) reveals that it is very similar to mel-34, differing significantly only towards the C terminus, and by virtue of an apparent deletion of 47 internal amino acid residues in the mouse sequence. Over the rest of the sequence the two proteins are 90% identical. It appears that mel-34 and Tyrs-33

represent the same (orthologous) gene in the two species, and pMT4 is encoded by a distinct but related gene. Given the biochemical evidence that tyrosinase is encoded at the albino locus I propose that pMT4 does not encode authentic tyrosinase but should be termed the tyrosinase-related protein (TRP) gene.

Genetic Mapping of the Mouse TRP Gene. The TRP gene is limited in its expression to melanocytes and melanoma (ref. 15; unpublished results). There was, therefore, a possibility that it would be encoded by a previously identified pigmentation gene. I determined its position in the mouse gene map by using RI strains. This analysis uses restriction fragment length variants between progenitor strains, which segregate through the RI strains and can be linked to known markers (22). A number of fragments from pMT4 were used to probe Southern blot hybridizations of RI progenitor strain DNAs digested with a number of restriction enzymes. Most probes derived from pMT4 hybridized to more than one fragment, indicating the presence of introns interrupting the coding sequence or the presence of cross-hybridizing sequences elsewhere in the genome. One probe, however, the 256-base-pair (bp) *Pvu* II fragment, nucleotides 682 to 943 of pMT4 (encoding amino acids 187 to 272), hybridized to a single 4.9-kilobase-pair (kb) *Taq* I fragment in the inbred strains DBA/2J (Fig. 1a), C57L/J, and BALB/cBy. This fragment must derive from the locus contained in pMT4. The strains C57BL/6/J (Fig. 1a) and AKR/J have two fragments of 3.7 and 1.2 kb, probably arising from the presence of an additional *Taq* I site. I refer to the fragment as seen in C57BL/6/J as the B haplotype and the fragments seen in DBA/2J as the D haplotype.

I determined the strain distribution pattern of the D and B haplotypes in 21 animals of the BXD RI series (Fig. 1b), 10 animals of the AKXL series, and 6 of the CXB series. In all 37 animals the B haplotype is concordant with the wild-type black (B) genotype and the D haplotype with the mutant brown (b) genotype (Table 1) (ref. 22; B. A. Taylor, personal

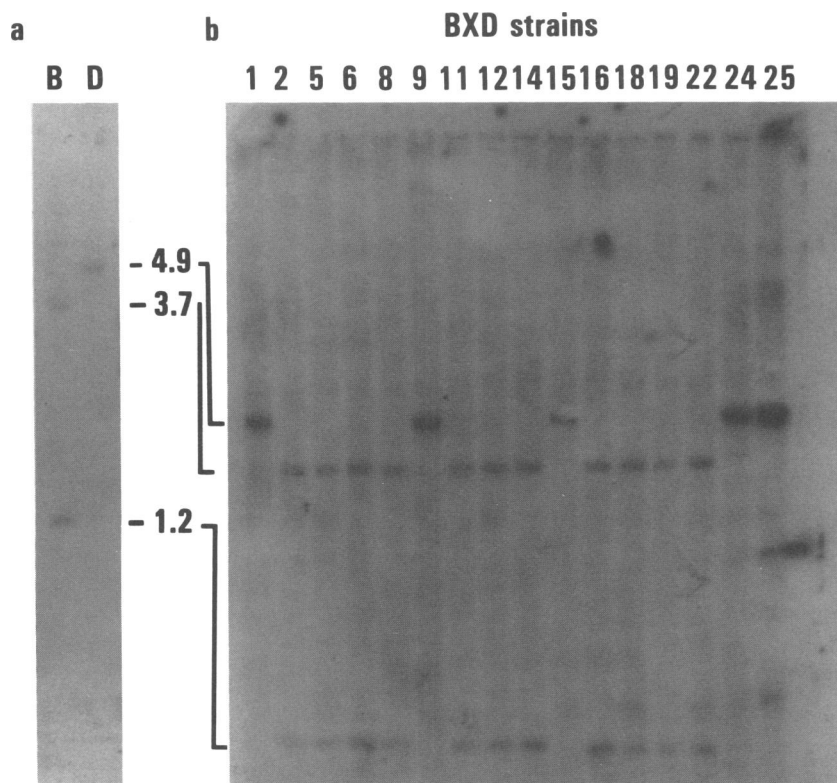


FIG. 1. Southern blot hybridizations. (a) *Taq* I digests of C57BL/6J (B) and DBA/2J (D) DNA, probed with the 256-bp *Pvu* II fragment of pMT4. (b) *Taq* I digests of DNA from 16 BXD RI strains, probed as for a. Sizes of the fragments are indicated in kb.

Table 1. Strain distribution pattern of D and B haplotypes correlated with strain distribution pattern of the *B* and *b* pigmentation genotypes

		BXD RI strains																AKXL RI strains										CXB RI strains										
		1	2	5	6	8	9	11	12	14	15	16	18	19	22	24	25	27	28	29	30	32	5	8	9	12	17	19	21	24	28	28	E	G	H	I	J	K
Haplotype	D	B	B	B	B	D	B	B	B	D	B	B	B	B	D	D	D	D	B	D	B	D	B	B	D	B	D	D	D	D	D	D	B	D	D	D	D	B
Genotype	<i>b</i>	<i>B</i>	<i>B</i>	<i>B</i>	<i>B</i>	<i>b</i>	<i>B</i>	<i>B</i>	<i>B</i>	<i>B</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>B</i>	<i>b</i>	<i>B</i>	<i>b</i>	<i>B</i>	<i>B</i>	<i>b</i>	<i>B</i>	<i>B</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>B</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>B</i>	<i>b</i>	<i>b</i>	<i>B</i>	

Haplotypes were determined by hybridization of *Taq* I digests with the 256-bp *Pvu* II probe of pMT4.

communication). The TRP gene therefore maps at or very close to the *b* locus. According to Silver (23), these data place the gene, at 95% confidence limits, within 2.8 centimorgans of the *b* locus.

Complete Linkage Disequilibrium Between the D Haplotype and the *b* Mutation. Further genetic data may imply a much closer linkage between TRP and *b*. There is complete linkage disequilibrium between the D haplotype and the *b* mutation, in that all *b* inbred strains examined, DBA/2J, C57L/J, BALB/cBy, ST/bJ, and NZW/LacJ (Fig. 2 and Table 2), as well as the PT stock and homozygous *b* mice of the random-bred Q stock (which segregate *b* within the colony) contain the D haplotype (Fig. 2). All *B* mice examined, including C57BL6/J, AKR/J, 101/H, C3H/HeH, 129, LP/J, and

CBA/Ca have the B haplotype (Table 2 and Fig. 2). These data are not quantifiable in terms of genetic distance, but they certainly suggest that the TRP gene is very close to, and probably identical with, the *B* locus.

A Cross-Hybridizing Fragment Maps Elsewhere in the Genome. An additional *Taq* I fragment variant is revealed by using a larger, ≈1.6-kb, *Hind*III fragment of pMT4 as probe. This probe spans bases 618 to 2186 and includes the *Pvu* II fragment used as probe in Fig. 1. It hybridizes to a number of *Taq* I fragments, including the D and B haplotype fragments (Fig. 2). The largest of these, a fragment of 12.8 kb, is present in all inbred strains except the related strains 129 and 101/H and strain NZW/LacJ, which have a 9-kb fragment (Table 2 and Fig. 2). The smaller fragment is also present in some mice of the Q stock. This variable fragment segregates independently of the D haplotype through the Q stock (Fig. 2). Mice Qb1 to Qb4 are all brown phenotype and have the D haplotype (4.9-kb fragment). However, Qb3 has the 12.8-kb fragment, Qb1 and Qb4 have the 9-kb variant, and Qb2 is heterozygous for both.

These fragments must represent a cross-hybridizing sequence mapping elsewhere in the genome. The location of this sequence is not currently known, nor is it known whether it is a functional gene or a pseudogene. It is not the tyrosinase gene at the albino locus, as it is present in the two albino deletion mutant mouse DNAs from which tyrosine is deleted (unpublished data).

Other Alleles of *B*. In addition to the old *b* mutation, there are several other alleles of *B* (1). DNAs from the spontaneous light mutation (*B^h*) (24) and from a radiation-induced cordovan mutation (*b^{ch}*) (4) have been examined and show no differences from wild-type DNA in the fragments hybridizing to probes spanning the whole of pMT4 (data not shown). Both mutations arose on a *B* background and are present within the context of the B haplotype.

DISCUSSION

The map location of the TRP gene, the tissue-restricted nature of its expression, and its similarity to tyrosinase strongly implicate TRP as the cause of the black/brown phenotype. This predicts that there are sequence differences

Table 2. Summary of fragments detected in inbred strains

<i>Taq</i> I fragment, kb	Inbred strains
12.8	DBA/2J, C57BL6/J, C57L/J, AKR/J, BALBc/By, ST/bJ, LP/J, C3H/HeH, CBA/Ca
9	NZW/LacJ, 101/H, 129
6.6	All these strains
4.9 (D)	DBA/2J, C57L/J, BALBc/By, ST/bJ, NZW/LacJ
3.7 (B)	C57BL6/J, AKR/J, 101/H, 129, LP/J, C3H/HeH, CBA/Ca
3.2	All these strains
2.65	All these strains
1.2 (B)	C57BL6/J, AKR/J, 101/H, 129, LP/J, C3H/HeH, CBA/Ca

Fragments sizes listed on the left are those detected in the *Taq* I digests of DNA from the inbred strains with the 1.6-kb *Hind*III probe of pMT4. D and B denote fragments of the D and B haplotypes.

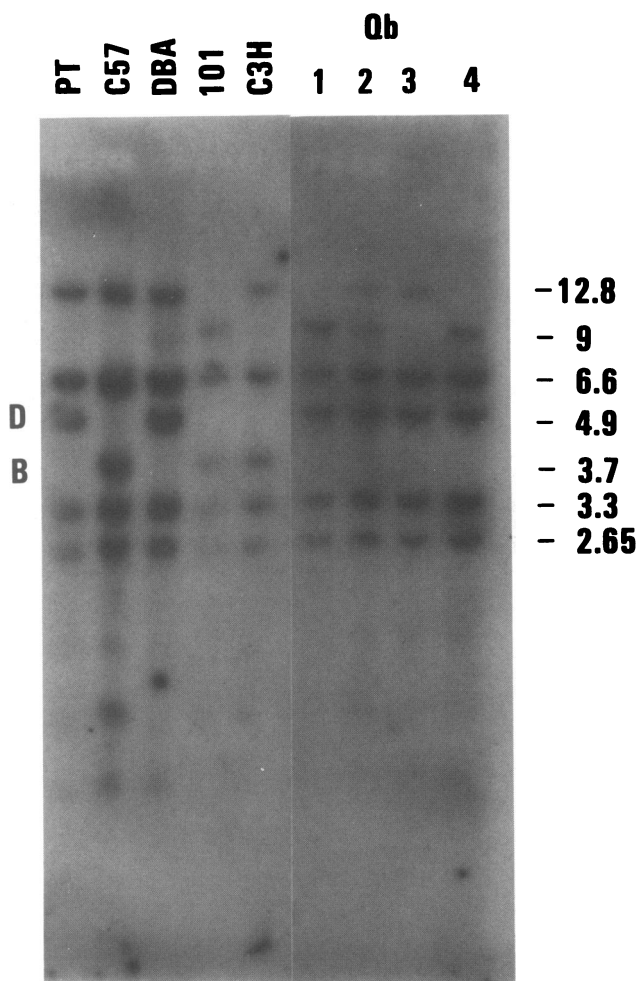


FIG. 2. Southern blot hybridization to *Taq* I digests of DNA from mice of several genotypes, probed with the 1.6-kb *Hind*III fragment of pMT4. Lanes: PT, Harwell PT stock; C57, C57BL6/J; DBA, DBA/2J; 101, 101/H; C3H, C3H/HeH; Qb1–4, homozygous *b* mice from the Mammalian Development Unit Q stock. Sizes of fragments are indicated on the right in kb, and the 4.9- and 3.7-kb fragments diagnostic of the D and B haplotypes are indicated on the left. Additional, more weakly hybridizing fragments can also be seen, of lengths 1.6, 1.1, and 0.85 kb.

between TRP genes from animals carrying different *b* locus alleles. It is important, then, to note that there is at least one amino acid difference between the protein encoded by the TRP gene of C57BL/6 animals (i.e., the *B* allele) and that encoded by the gene from BALB/c animals (the *b* mutation) (S. Shibahara, personal communication).

There is concordance between both tyrosinase enzymic activity and immunological reactivity and *c* locus genotype (13, 14), and it is likely that the cDNAs mapping at the *c* locus (11, 12) encode authentic tyrosinase. By contrast, tyrosinase activity in *b* mutant skin slices is actually higher than in wild-type skin (1, 13, 25), indicating that tyrosinase is not encoded at the *b* locus. Similarities of sequence and intracellular location may explain the independent misidentification by two workers of TRP clones as tyrosinase. It should, however, be noted that the serum used by Kwon *et al.* to isolate human tyrosinase cDNA is the same antiserum to hamster tyrosinase (RA5) used in this study.

The Function of TRP. Assuming TRP is the product of the *b* locus, what can be inferred of its function? As the *b* mutation is likely to be the null allele, its wild-type function cannot be essential for pigmentation, only for production of black rather than brown pigment. The extensive similarity to tyrosinase suggests that it may have an enzymatic function. In particular, the conserved positions of histidine and cysteine residues (12) might reflect binding of metal ion cofactors. There are enzymes genetically distinct from tyrosinase involved in melanin synthesis, in particular dopachrome conversion factor or dopachrome oxidoreductase (DCOR) (26–28). Eumelanin can be formed from a range of precursors, each derived from the other. Almost all steps of this pathway are catalyzed by one of several activities of tyrosinase, or are nonenzymatic, except the conversion of dopachrome to 5,6-dihydroxyindole, which is accelerated by DCOR. If one proposed that TRP was DCOR, one might speculate that the eumelanin synthesized from those substrates made without DCOR is brown, while that made from the full wild-type complement of substrates is black.

On the other hand, morphological studies suggest a role for the *b* locus product in maintaining melanin granule structure. A comprehensive analysis (29) of granule structure in *B* and *b* melanocytes has revealed that *B* granules are extended ovoids and contain few membranes, arranged in a simple rolled manner, whereas *b* granules are spherical and contain many membranous strands in a tangled mass.

It is possible that the protein could, directly or indirectly, perform both an enzymatic and a structural role. Possibly a melanosomal enzyme has been recruited for such a dual role, or the product of the enzymatic action of TRP might perform the structural task. Perhaps the dense black eumelanin affects melanosome morphology in a different way from the more diffuse brown pigment. It is also possible that at one time in evolution tyrosinase itself had a dual enzymatic/structural role, which, following gene duplication, became divided between two homologous proteins. Both tyrosinase and TRP have a signal sequence and a transmembrane region; both are presumably integral proteins of the melanin granule membrane. The sequence of TRP from mice carrying *b* and other mutant alleles will no doubt cast light on its function.

Origins of the *b* Mutation. It is not known whether the *Taq* I polymorphism that results in the D haplotype causes the brown phenotype. It is not obligatory, however, as induced *b* mutations do not have the *Taq* I change (I.J.J. and Jack Favor, unpublished data). Equally, the D haplotype is never associated with the black phenotype. The likeliest explanation for the association seen in laboratory strains is that all *b* mutations on the D haplotype derive from one mutational event.

The history of the inbred laboratory mouse strains has been well documented (30, 31). Many strains can be traced back to a small group of workers in the United States earlier this

century, and it is not surprising, therefore, that DBA/2J, C57L/J, and BALB/cBy carry the same *b* mutation. More notable is the presence of the same mutation in the ST/b strain, which originated from outbred mice in Denmark, and in the NZW/LacJ strain, derived from an outbred population in England. Most laboratory mice came from mice kept as pets by mouse fanciers, between whom animals were distributed. No doubt the activities of mouse fanciers spread the *b* mutation around the world.

The origin of the less common 9-kb *Taq* I fragment, which maps elsewhere, is not so easily discerned. NZW, 101, and 129 mice have the variant fragment. Two of these, 101 and 129, share ancestry in Dunn's laboratory in 1928, where stocks from England were used as source of genetic material. Interestingly, strain LP/J has the same roots as 101 and 129 but does not have the 9-kb *Taq* I fragment. The origin of NZW from English stocks might suggest a common progenitor with 101 and 129, but it is quite possible that the 9-kb fragment represents a frequent polymorphism in the wild that has been fixed independently twice.

Specific-Locus Mutation Experiments. The TRP probe will allow *b* mutations arising in specific-locus experiments to be studied. Until now, there has been no way that a newly induced *b* mutation could be distinguished from the old *b* mutation in the tester stock used to reveal it, and as a result such new mutations have often been discarded. As new mutations are produced on a B haplotype it will now be more straightforward to keep track of the mutation in later generations. It will also be possible to see rapidly if the new mutation is associated with a deletion, and if so it would be worthwhile testing it for a recessive pleiotropic effect.

The difficulties in working with new *b* mutations have meant that almost no information is available as regards nearby developmental genes. One possible exception is dominant reduced ear (32), an x-ray-induced mutation in which a recessive *b* mutation occurred simultaneously with a dominant small-ear mutation. Although now extinct, this was probably a deletion that produced the reduced ear phenotype through haploinsufficiency of a gene linked to *b*. There is much potential for study of developmental genetics in this region of the genome.

I thank all colleagues who generously gave materials as noted in the text, in particular Shige Shibahara for the use of pMT4 and communicating unpublished data and Byoung Kwon for communicating results prior to publication. I also thank Anne McLaren, Robin Lovell-Badge, Peter Little, Yvonne Edwards, and Nick Hastie for useful discussions. Financial support has been provided by the Medical Research Council and the Lister Institute for Preventive Medicine. I.J.J. is a Lister Institute Research Fellow.

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